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PATENT COOPERATION TREATY

1641

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

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ASTRAZENECA AB
Intellectual Property, Patents
S-151 85 Södertälje
SUÈDE

Date of mailing (day/month/year) 06 October 2000 (06.10.00)	
Applicant's or agent's file reference R 1944-1 WO	**IMPORTANT NOTIFICATION**
International application No. PCT/SE99/00749	**International filing date** (day/month/year) 04 May 1999 (04.05.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
PRAHLAD, Dwarakanath Astra Biochemicals Pvt. Ltd. P.O. Box 8013 Malleswaram Bangalore 560080 India	IN	IN
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
PRAHLAD, Dwarakanath AstraZeneca R&D Bangalore P.O. Box 8013 Sadashivnagar Bangalore 560080 India	IN	IN
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[ ] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Ellen Moyse
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

003569107

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PCT/SE99/00749

PATENT COOPERATION TREATY

09/341196

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From the INTERNATIONAL BUREAU

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

ASTRAZENECA AB
Intellectual Property, Patents
S-151 85 Södertälje
SUÈDE

Date of mailing (day/month/year)	IMPORTANT NOTIFICATION
03 April 2000 (03.04.00)	
Applicant's or agent's file reference R 1944-1 WO	
International application No. PCT/SE99/00749	**International filing date** (day/month/year) 04 May 1999 (04.05.99)

1. The following indications appeared on record concerning:

[X] the applicant [ ] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
ASTRA AKTIEBOLAG S-151 85 Södertälje Sweden	SE	SE
	Telephone No. +46 8 553 260 00	
	Facsimile No. +46 8 553 288 20	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [X] the name [ ] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
ASTRAZENECA AB S-151 85 Södertälje Sweden	SE	SE
	Telephone No. +46 8 553 260 00	
	Facsimile No. +46 8 553 288 20	
	Teleprinter No.	

3. Further observations, if necessary:
Please note that the above change also refers to the name indicated in Box No. IV of the request form.

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer A. Karkachi
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

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PATENT COOPERATION TREATY

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NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 25 January 2000 (25.01.00)	
International application No. PCT/SE99/00749	**Applicant's or agent's file reference** R 1944-1 WO
International filing date (day/month/year) 04 May 1999 (04.05.99)	**Priority date** (day/month/year) 15 May 1998 (15.05.98)
Applicant DESOUSA, Sunita et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

14 December 1999 (14.12.99)

[ ] in a notice effecting later election filed with the International Bureau on:

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2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer R. E. Stoffel
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

3067554

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

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NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

ASTRAZENECA AB
Intellectual Property, Patent
S-151 85 Södertälje
SUÈDE

Date of mailing (day/month/year) 06 October 2000 (06.10.00)	
Applicant's or agent's file reference R 1944-1 WO	**IMPORTANT NOTIFICATION**
International application No. PCT/SE99/00749	**International filing date** (day/month/year) 04 May 1999 (04.05.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address	State of Nationality	State of Residence
DESOUSA, Sunita Astra Biochemicals Pvt. Ltd. P.O. Box 8013 Malleswaram Bangalore 560080 India	IN	IN
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address	State of Nationality	State of Residence
DESOUSA, Sunita AstraZeneca R&D Bangalore P.O. Box 8013 Sadashivnagar Bangalore 560080 India	IN	IN
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

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The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Ellen Moyse
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

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Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
R 1944-1 WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/SE99/00749	04.05.1999	15.05.1998

International Patent Classification (IPC) or national classification and IPC7

C 12 Q 1/48, C 12 M 1/34

Applicant

AstraZeneca AB et al

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of _____ sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
14.12.1999	22.08.2000

Name and mailing address of the IPEA/SE

Patent- och registreringsverket
Box 5055
S-102 42 STOCKHOLM

Telex
17978
PATOREG-S

Facsimile No. 08-667 72 88

Authorized officer

Carolina Palmcrantz/gh

Telephone No. 08-782 25 00

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SE99/00749

I. Basis of the report

1. This report has been drawn on the basis of *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.)*:

☒ the international application as originally filed.

☐ the description, pages _____, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____.

☐ the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. _____, filed with the letter of _____,
Nos. _____, filed with the letter of _____.

☐ the drawings, ~~sheets~~/fig _____, as originally filed,
~~sheets~~/fig _____, filed with the demand
~~sheets~~/fig _____, filed with the letter of _____,
~~sheets~~/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☐ the description, pages _____
☐ the claims, Nos. _____
☐ the drawings, ~~sheets~~/fig _____

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the supplemental Box (Rule 70.2(c)).

4. Additional observations, if necessary:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/SE99/00749

V. Resoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	2-9	YES
	Claims	1	NO
Inventive step (IS)	Claims		YES
	Claims	1-9	NO
Industrial applicability (IA)	Claims	1-9	YES
	Claims		NO

2. Citations and explanations

The present application concerns a method for detecting peptidoglycan synthesis, in order to e.g. screen for antibacterial compounds. The method is based on a Scintillation Proximity Assay (SPA). By using SPA the method can be performed entirely in solution.

The International Search Report revealed five documents of importance:

D1) EP 0890644 A2 (SMITHKLINE BEECHAM CORPORATION), 13 January 1999 (13.01.99), page 2, lines 35-36; and page 17, lines 16-21

D2) Dialog Information Service, File 5, Biosis, Dialog accession no. 11619936, Biosis accession no. 199800401917, Eid Clark et al: "Synthesis of a radioiodinated park nucleotide analog: A new tool for antibacterial screen development", Journal of Labelled Compounds and Radiopharmaceuticals 41 (8):p 705-716 Aug., 1998

D3) WO 9615258 A1 (THE UPJOHN COMPANY), 23 May 1996 (23.05.96), see page 2, lines 7-28; and page 4, lines 10-11, 15

D4) Drug discovery today, Volume 1, No 7, July 1996, Neil D. Cook, "Scintillation proximity assay: a versatile high-througput screening technology" page 287 - page 294

D5) WO 9426413 A1 (AMERSHAM INTERNATIONAL PLC), 24 November 1994 (24.11.94), abstract

.../...

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: V

D1 and D2 are published after the priority date of the present application. D1, however, is considered to be relevant against novelty.

D1 discloses the MurA gene from Staphylococcus aureus encoding DP-N-Acetylglucosamine enolpyruvyl transferase. The enzyme catalyses the first step of peptidoglycan biosynthesis. A Scintillation Proximity Assay may be used to characterize the interaction between a MurA polypeptide with another MurA polypeptide or a different polypeptide. Therefore, since the transferase is considered to be a measure of a coming peptidoglycan synthesis, claim 1 of the present application is not considered to be novel in relation to D1.

D3 pertains to a Scintillation Proximity Assay (SPA) for detecting the presence of N-acetylgalactosaminyltransferase (GalNac-transferase) (see page 3, line 31-page 4, line 8). The assay can be used for screening for compounds affecting GalNac-transferase activity. GalNac-transferase is an intracellular membrane bound enzyme believed to be involved in the secretory pathway (see page 2, lines 15-16 and 29-31).

D4 concerns the use of the Scintillation Proximity Assay as a tool for high-throughput screening for a wide variety of biochemical and cellular targets (see the abstract).

D5 discloses an apparatus for studying cellular processes by Scintillation Proximity Assay.

Therefore, since SPA is known in the prior art to be useful in studying cellular processes such as the presence of the membrane bound enzyme GalNac-transferase, it is considered to be obvious to a person skilled in the art to use SPA also for detecting peptidoglycan synthesis, e.g. transglycosylase or transpeptidase. Consequently, claims 1-9 are not considered to involve an inventive step.

09/341196

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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C12Q 1/48, C12M 1/34

A1

(11) International Publication Number: **WO 99/60155**

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(21) International Application Number: PCT/SE99/00749

(22) International Filing Date: 4 May 1999 (04.05.99)

(30) Priority Data:

1019/MAS/98	15 May 1998 (15.05.98)	IN
9802210-6	22 June 1998 (22.06.98)	SE

(71) Applicant *(for all designated States except US)*: ASTRA AKTIEBOLAG [SE/SE]; S-151 85 Södertälje (SE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DESOUSA, Sunita [IN/IN]; Astra Biochemicals Pvt. Ltd., P.O. Box 8013, Malleswaram, Bangalore 560080 (IN). PRAHLAD, Dwarakanath [IN/IN]; Astra Biochemicals Pvt. Ltd., P.O. Box 8013, Malleswaram, Bangalore 560080 (IN).

(74) Agent: ASTRA AKTIEBOLAG; Intellectual Property, Patents, S-151 85 Södertälje (SE).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: A SCINTILLATION PROXIMITY ASSAY FOR THE DETECTION OF PEPTIDOGLYCAN SYNTHESIS

(57) Abstract

The invention provides a scintillation proximity assay for detecting peptidoglycan synthesis. The assay is especially suitable for high throughput screening of compounds affecting peptidoglycan synthesis.

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A SCINTILLATION PROXIMITY ASSAY FOR THE DETECTION OF PEPTIDOGLYCAN SYNTHESIS

The present invention relates to a new assay for detecting peptidoglycan synthesis.

Peptidoglycan is a major component of the bacterial cell wall that gives the wall its shape and strength. It is unique to bacteria and found in all bacteria, both gram-positive and gram-negative. Peptidoglycan is a polymer of glycan strands that are cross-linked through short peptide bridges. It consists of alternating β1-4 linked residues of *N*-acetyl glucosamine (Glc*N*Ac) and *N*-acetyl muramic acid (Mur*N*Ac). A pentapeptide chain is attached to Mur*N*Ac (Mur*N*Ac-pentapeptide) and cross-linking occurs between these peptide chains.

Biosynthesis of peptidoglycan can be divided into three stages: firstly, synthesis of the precursors in the cytoplasm, secondly, transfer of the precursors to a lipid carrier molecule and, thirdly, insertion of the precursors into the cell wall and coupling to existing peptidoglycan.

The precursors synthesised in the cytoplasm are the sugar nucleotides: UDP-*N*-acetyl-glucosamine (UDP-Glc*N*Ac) and UDP-*N*-acetylmuramylpentapeptide (UDP-Mur*N*Ac-pentapeptide).

The second stage, which occurs in the cytoplasmic membrane, is catalysed by two enzymes and involves synthesis of a disaccharide unit on a lipid carrier, undecaprenyl phosphate. The lipid carrier is also involved in the synthesis of other components of the bacterial cell wall.

The first enzyme catalyses the transfer of phosphoryl-*N*-acetyl muramyl pentapeptide from UDP-Mur*N*Ac-pentapeptide to undecaprenol phosphate with the simultaneous release of UMP. This enzyme is called phospho-*N*-acetylmuramyl-pentapeptide translocase (hereafter referred to as "the translocase") and is the product of the gene mraY in

Escherichia coli. The product, undecaprenol-pyrophosphate-N-acetylmuramylpentapeptide (Lipid-P-P-MurNAc-pentapeptide) or Lipid I or Lipid linked precursor I is the substrate for the second enzyme.

N-acetylglucosaminyl transferase, transfers N-acetylglucosamine from UDP-GlcNAc (with simultaneous release of UDP) to form undecaprenol-pyrophosphoryl-*N*-acetylmuramylpentapeptide-*N*-acetylglucosamine or Lipid II or Lipid linked precursor II. This enzyme is also called UDP-*N*-acetylglucosamine: *N*-acetylmuramyl(pentapeptide)-P-P-undecaprenol-*N*-acetylglucosamine transferase (hereafter referred to as "the transferase"). The enzyme is the product of the gene murG in *Escherichia coli*.

The translocase and the transferase enzymes are essential for bacterial viability (see respectively D.S. Boyle and W.D. Donachie, J. Bacteriol. (1998), **180**, 6429-6432 and D. Mengin-Lecreulx, L. Texier, M. Rousseaue and J. Van Heijenoot, J. Bacteriol. (1991), **173**, 4625-4636).

In the third stage, at the exterior of the cytoplasmic membrane, polymerisation of the glycan occurs. The disaccharide-pentapeptide unit is transferred from the lipid carrier to an existing disaccharide unit or polymer by a peptidoglycan transglycosylase (also referred to as a peptidoglycan polymerase) (hereafter referred to as "the transglycosylase"). The joining of the peptide bridge is catalyzed by peptidoglycan transpeptidase (hereafter referred to as "the transpeptidase"). Both enzyme activities which are essential reside in the same molecule, the penicillin binding proteins (or PBPs), as in PBP 1a or 1b in *Escherichia coli*. These are the products of the ponA and ponB genes respectively, in *Escherichia coli*.

On transfer of the disaccharide-pentapeptide unit from the lipid precursor to an existing peptidoglycan chain the lipid is released as a molecule of undecaprenol pyrophosphate. This has to be cleaved by a bacitracin-sensitive undecaprenyl pyrophosphorylase, also called undecaprenol pyrophosphorylase or C55-isoprenyl

pyrophosphorylase (hereafter referred to as the "lipid pyrophosphorylase") to generate undecaprenol phosphate which can then re-enter the cycle at the second stage. Since inhibition of this enzyme will inhibit recycling of the lipid precursor it could also inhibit formation of peptidoglycan.

The transglycosylase is usually assayed by radiolabelling one of the sugar molecules and monitoring its incorporation into peptidoglycan. It is a difficult enzyme to assay because the lipid carrier molecule with bound disaccharide is neither simple to make nor water-soluble and, furthermore, the reaction only occurs on a solid phase (e.g. on Whatman 3 mm paper) and so the reaction conditions are difficult to control.

The transglycosylase activity may alternatively be assayed indirectly in a solution phase assay which, whilst being easier to control, requires the use of three of the other key enzymes involved in peptidoglycan synthesis, the translocase (e.g. the mraY gene product), the transferase (e.g. the murG gene product) and the lipid pyrophosphorylase.

In both types of assay, quantification of the products of enzymatic reaction is carried out using paper chromatography in which peptidoglycan stays at the origin and the reactants move away from the origin.

It would be desirable to develop an assay for detecting peptidoglycan synthesis which dispensed with the need for paper chromatography altogether. More particularly, it would be desirable to develop an assay for detecting peptidoglycan synthesis in which the reaction and quantification of the products of reaction could be performed entirely in the solution phase, for example, in a microtitre plate.

In accordance with the present invention, there is therefore provided an assay for detecting peptidoglycan synthesis, which comprises the steps of:

(1) incubating a reaction mixture comprising in aqueous medium a UDP-*N*-acetylmuramylpentapeptide (UDP-Mur*N*Ac-pentapeptide), radiolabelled UDP-*N*-acetyl

glucosamine (UDP-Glc*N*Ac), a source of divalent metal ions, a source of undecaprenyl phosphate, a source of peptidoglycan, a source of translocase enzyme (e.g. the *E.coli* mraY gene product), a source of transferase enzyme (e.g. the *E.coli* murG gene product), a source of transglycosylase enzyme, a source of transpeptidase enzyme (e.g. *E. coli* PBP 1a or PBP 1b) and a source of lipid pyrophosphorylase, under conditions suitable for peptidoglycan synthesis;

(2) adding a divalent metal ion chelator compound to the reaction mixture of step (1);

(3) adding lectin-coated beads impregnated with a fluorescer to the reaction mixture of step (2); and

(4) measuring light energy emitted by the fluorescer.

In the context of the present specification, it should be understood that the abbreviation "UDP" refers to uridine (5'-)diphosphate.

The assay according to the present invention is very conveniently carried out on 96-well microtitre plates, thereby enabling a fast, simple and reproducible way of measuring peptidoglycan synthesis.

In step (1), the UDP-Mur*N*Ac-pentapeptide used may be any of those usually present in naturally-occurring peptidoglycans and is conveniently purified from bacteria or made enzymatically with precursors from bacteria, e.g. by methods similar to that described by T. den Blaauwen, M. Aarsman and N. Nanninga, J. Bacteriol. (1990), **172**, 63-70). A preferred UDP-Mur*N*Ac-pentapeptide to use is UDP-Mur*N*Ac-L-alanine-γ-D-glutamic acid-m-diaminopimelic acid-D-alanine-D-alanine from *Bacillus cereus*. The purified UDP-Mur*N*Ac-pentapeptide may also contain a certain amount of the tripeptide and tetrapeptide analogues and these may also participate effectively in the peptidoglycan synthesis reaction.

The concentration of UDP-Mur*N*Ac-pentapeptide used will typically be in the range from 50μM, preferably from 75μM, to 300μM, preferably 200μM, more preferably 100μM, per well of the microtitre plate.

As radiolabelled UDP-*N*-acetyl glucosamine, it is convenient to use tritiated UDP-*N*-acetyl glucosamine (UDP-[3H]Glc*N*Ac, commercially available from NEN-Dupont), preferably in a concentration of from 0.25 to 25μM per well of the microtitre plate, with radioactivity in the range from, e.g., 0.07 μCi to 2.00 μCi per well, preferably from 0.10 μCi to 1.00 μCi per well, and more preferably from 0.10 μCi to 0.5 μCi per well.

The divalent metal ions used are preferably magnesium ions. A suitable source of magnesium ions is magnesium chloride.

The membranes of *Escherichia coli* bacteria may conveniently be used and indeed are preferred as a source of undecaprenyl phosphate, peptidoglycan, translocase enzyme, transferase enzyme, transglycosylase enzyme, transpeptidase enzyme and lipid pyrophosphorylase enzyme. The quantity of membranes used will typically be in the range from 1 to 20μg, particularly from 4 to 6μg, protein per well of the microtitre plate. The membranes may be prepared by methods known in the art.

The aqueous medium used in step (1) is preferably a buffer solution, e.g. of Tris[hydroxymethyl]aminomethane hydrochloride ("Tris-HCl"), having a pH of about 7.5. Tris-HCl is commercially available from the Sigma-Aldrich Co. Ltd.

If the assay is intended to be used as a screen for identifying anti-bacterial compounds that are antagonists of the translocase, transferase, transglycosylase, transpeptidase or lipid pyrophosphorylase enzymes, the reaction mixture of step (1) may further comprise one or more test compounds in varying concentrations. Since the transglycosylase and transpeptidase enzymes are essential for bacterial growth and are located on the cell surface, these enzymes are regarded as especially good targets for the development of anti-

bacterial drugs as the drugs would not need to enter the bacterial organism through the cell wall and therefore the problems of cell wall permeability and also drug resistance brought about by changes in cell wall permeability are avoided.

The reaction mixture of step (1) is maintained at a temperature at or about 37 °C for a period of 0.5 to 4 hours, e.g. 1.5 hours, under conditions suitable for peptidoglycan synthesis to occur.

Peptidoglycan synthesis is terminated in step (2) by the addition of a suitable amount of a divalent metal ion chelator compound, e.g. ethylenediaminetetraacetic acid (EDTA) which is commercially available from the Sigma-Aldrich Co. Ltd. The concentration of the chelator compound will of course depend on the particular chelator compound used and should be sufficient to chelate all the divalent metal ions; in the case of EDTA the concentration will typically be about 15 mM per well of the microtitre plate.

In step (3), preferred lectin-coated beads impregnated with a fluorescer to use are those described in US Patent No. 4,568,649 and European Patent No. 154,734. The beads (known as "Scintillation Proximity Assay" (or SPA) beads) are commercially available from Amersham Inc. Most preferred are wheatgerm agglutinin-coated SPA beads which are capable of binding sugar molecules, specifically N-acetyl glucosamine. Thus, through the binding of N-acetyl glucosamine to the SPA beads, radiolabelled peptidoglycan formed in step (1) is brought into close proximity with the fluorescer which becomes activated by the radiation energy, resulting in the emission of light energy which is subsequently measured in step (4).

The beads which are conveniently added in the form of an aqueous suspension are contacted with the reaction mixture of step (2) for a period of 3 hours or more (e.g. overnight) before the plate is "counted" in step (4), e.g., in a "Microbeta Tilux" counter.

Apart from screening for anti-bacterial compounds as mentioned above, the assay according to the invention may, since it is sensitive to β-lactam antibiotics, be used alternatively to screen for novel β-lactams and also to measure the concentration of β-lactam antibiotics or to measure the activity of β-lactamases, enzymes that degrade β-lactams. In this way, the assay can be used as a diagnostic to detect disease-causing bacteria that are resistant to β-lactams because of the production of β-lactamases. Further, the assay may be used to identify inhibitors of β-lactamases, a key area of drug development.

The present invention will be further illustrated with reference to the following Example.

Example 1

(i) The wells of a microtitre plate were individually filled with a total volume of 25 µl of a reaction mixture comprising an aqueous buffer solution of 100 mM Tris[hydroxymethyl]aminomethane hydrochloride ("Tris-HCl") and 10 mM magnesium chloride (pH 7.5), 75 µM UDP-Mur*N*Ac-L-alanine-γ-D-glutamic acid-m-diaminopimelic acid-D-alanine-D-alanine, 2.5 µM tritiated UDP-*N*-acetyl glucosamine (0.5 µCi per well), 4 µg of *Escherichia coli* AMA1004 cell membranes and a solution of test compound (e.g. Tunicamycin, Vancomycin, Moenomycin, Penicillin G, Ampicillin, Cephaloridine and Bacitracin) of varying concentration in 4% dimethylsulphoxide. Tunicamycin is a known antagonist of the translocase enzyme, Vancomycin and Moenomycin are known antagonists of the transglycosylase enzyme, Penicillin G, Ampicillin and Cephaloridine are known antagonists of the transpeptidase enzyme and Bacitracin is a known antagonist of the lipid pyrophosphorylase.

Four wells of the microtitre plate were used as controls: two wells contained no UDP-*N*-acetylmuramylpentapeptide (0% reaction controls) and a further two wells contained no test compound (100% reaction controls).

The E. coli membranes were prepared in the following manner.
Four to five colonies of the bacteria from an LB (Luria Bertani medium) agar plate were inoculated into 5 ml LB-broth and grown during the day (for 6-8 hours) at 37°C. In the evening 0.5 ml of this culture was used to inoculate 500 ml of LB-broth in a 2 l flask. The flask was incubated on a shaker at 30°C overnight; typically an A600 of 2.0-2.5 was reached. Early the next morning this culture was used to inoculate 6 l of LB-broth (using 500 ml of LB-broth per 2 l flask) such that the starting A600 was 0.4-0.6. The culture was grown for 2 hours at 37°C with vigorous shaking/aeration; the A600 reached was between 1.4 and 2.0. At this point the bacteria were cooled on ice and pelleted by centrifugation at 5,000 x g for 15 minutes. The cell pellet was washed with 500 ml of Buffer A (50 mM Tris-HCl, pH 7.5 / 0.1 mM $MgCl_2$) and resuspended in a minimal volume (< 20ml) of Buffer A. The cells were lysed using the French Pressure cell. The cell lysate was spun at 3,500 x g for 45 minutes. The supernatant was collected, diluted to 100 ml with Buffer A and ultra-centrifuged at 150,000 x g for 45 minutes. The pellet from this spin was washed by resuspending it in 100 ml of Buffer A and re-centrifuging at 150,000 x g for 30 minutes. This pellet was gently resuspended in a minimal volume (5-10 ml for 6 l culture) of Buffer A and frozen and stored in aliquots at -70°C. This is termed the membrane preparation and was used in the assay as a source of the peptidoglycan, five enzymes and undecaprenyl phosphate.

The microtitre plate was incubated at 37 °C for 1.5 hours and thereafter 5 µl of ethylenediaminetetraacetic acid (EDTA) was added to give a final EDTA concentration of 15 mM.

(ii) After addition of the EDTA, 170 µl of an aqueous suspension of wheatgerm agglutinin-coated scintillation proximity assay beads comprising 500 µg beads in a solution of Tris-HCl, pH 7.4, and t-octylphenoxypolyethoxyethanol ("Triton X-100", commercially sold by the Sigma-Aldrich Co. Ltd.) was added to each well such that the final concentration of Tris-HCl was 100 mM and that of Triton X-100 was 0.05%.

The plate was left for 3 hours at room temperature before being counted in the "Microbeta Trilux" counter.

Figure 1 is a graph showing the counts per minute (cpm) versus time based on the readings taken from the 100% controls.

Figure 2 is a graph showing the percentage inhibition of translocase (and thus peptidoglycan synthesis) versus Tunicamycin concentration.

Figure 3 is a graph showing the percentage inhibition of transglycosylase (and thus peptidoglycan synthesis) versus Vancomycin concentration.

Figure 4 is a graph showing the percentage inhibition of transglycosylase (and thus peptidoglycan synthesis) versus Moenomycin concentration.

Figure 5 is a graph showing the percentage inhibition of transpeptidase (and thus peptidoglycan synthesis) versus Penicillin G concentration.

Figure 6 is a graph showing the percentage inhibition of transpeptidase (and thus peptidoglycan synthesis) versus Ampicillin concentration.

Figure 7 is a graph showing the percentage inhibition of transpeptidase (and thus peptidoglycan synthesis) versus Cephaloridine concentration.

Figure 8 is a graph showing the percentage inhibition of lipid pyrophosphorylase (and thus peptidoglycan synthesis) versus Bacitracin concentration.

CLAIMS

1. A Scintillation Proximity Assay (SPA) for the detection of peptidoglycan synthesis.

2. An assay for detecting peptidoglycan synthesis, which comprises the steps of:
(1) incubating a reaction mixture comprising in aqueous medium a UDP-*N*-acetylmuramylpentapeptide, radiolabelled UDP-*N*-acetyl glucosamine, a source of divalent metal ions, a source of undecaprenyl phosphate, a source of peptidoglycan, a source of translocase enzyme, a source of transferase enzyme, a source of transglycosylase enzyme, a source of transpeptidase enzyme and a source of lipid pyrophosphorylase enzyme, under conditions suitable for peptidoglycan synthesis;
(2) adding a divalent metal ion chelator compound to the reaction mixture of step (1);
(3) adding lectin-coated beads impregnated with a fluorescer to the reaction mixture of step (2); and
(4) measuring light energy emitted by the fluorescer.

3. An assay according to claim 2, wherein the UDP-*N*-acetylmuramylpentapeptide is UDP-Mur*N*Ac-L-alanine-γ-D-glutamic acid-m-diaminopimelic acid-D-alanine-D-alanine.

4. An assay according to claim 2 or claim 3, wherein bacterial cell membranes represent a source of one or more of undecaprenyl phosphate, peptidoglycan, translocase enzyme, transferase enzyme, transglycosylase enzyme, transpeptidase enzyme and lipid pyrophosphorylase enzyme.

5. An assay according to claim 4, wherein the bacterial cell membranes are from *Escherichia coli*.

6. An assay according to any one of claims 2 to 6, wherein the reaction mixture of step (1) further comprises a test compound.

7. An assay according to claim 6, wherein the test compound is an antagonist of one of the enzymes.

8. An assay according to any one of claims 2 to 7, wherein ethylenediaminetetraacetic acid is used as the divalent metal ion chelator compound in step (2).

9. An assay according to any one of claims 2 to 8, wherein the lectin-coated beads comprise wheatgerm agglutinin.

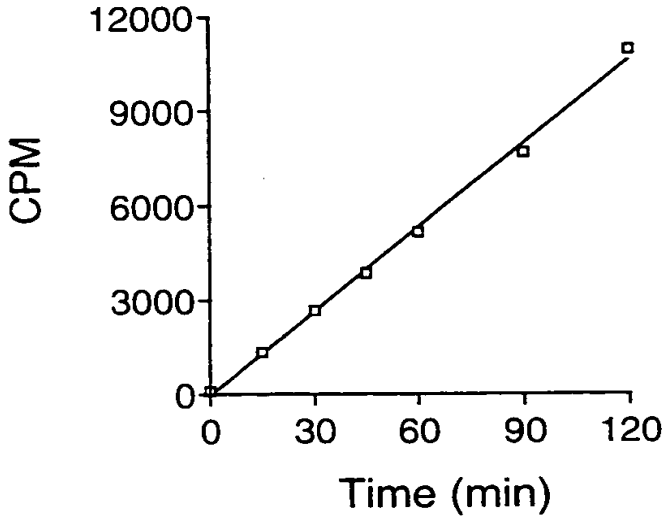

Figure 1

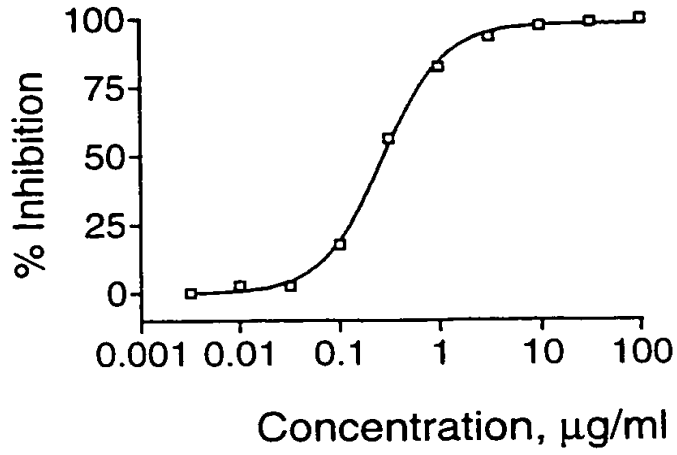

Figure 2

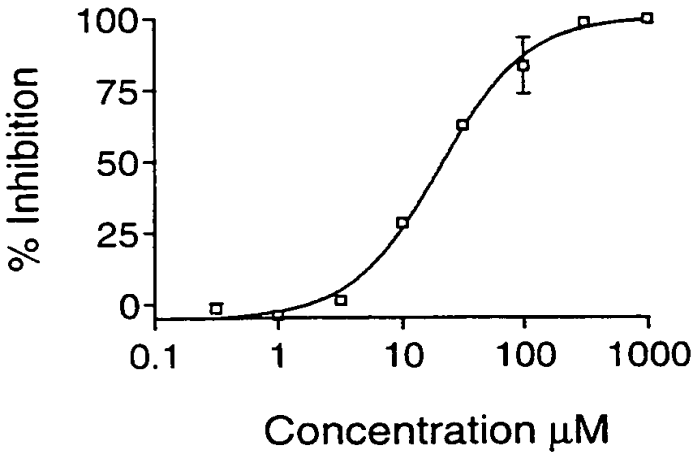

Figure 3

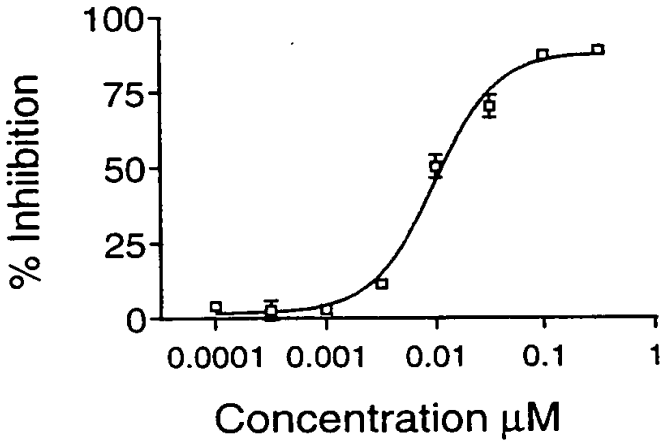

Figure 4

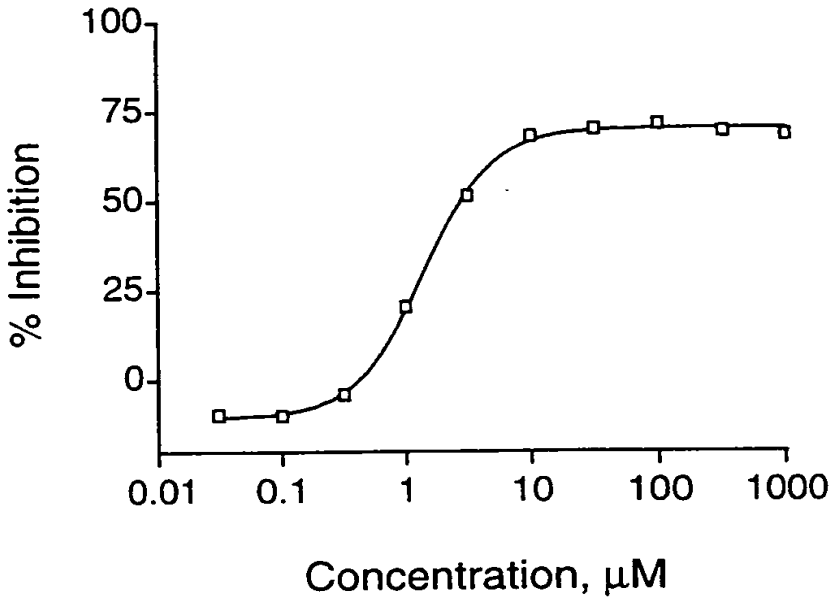

Figure 5

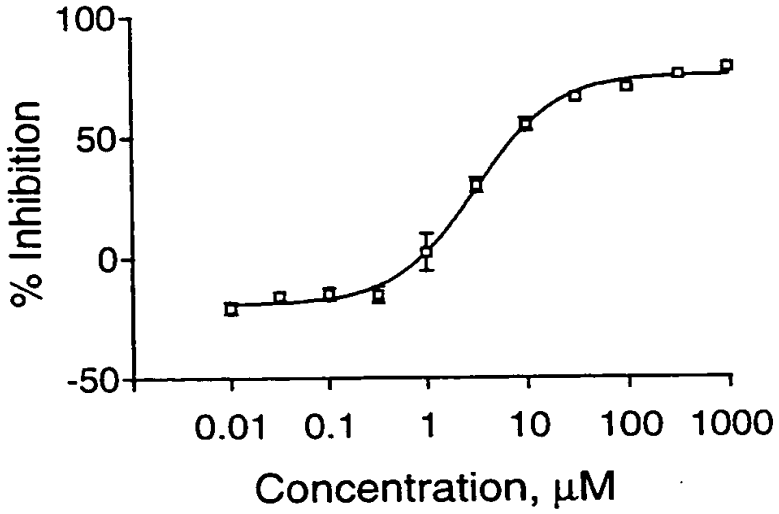

Figure 6

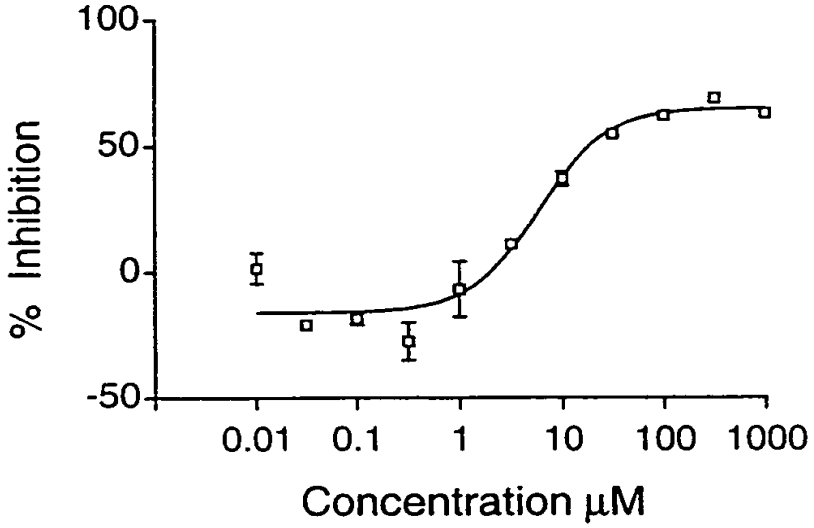

Figure 7

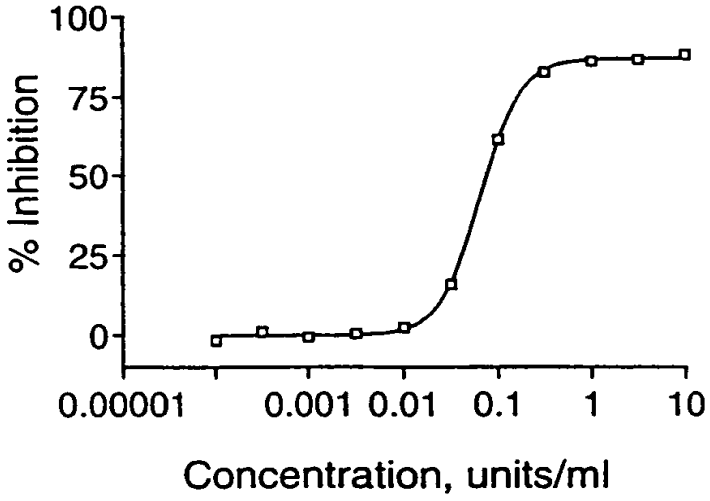

Figure 8

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* R 1944-1 WO

Box No. I TITLE OF INVENTION

NEW ASSAY

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Astra Aktiebolag
S-151 85 Södertälje
Sweden

☐ This person is also inventor.

Telephone No.
+46 8 553 260 00

Facsimile No.
+46 8 553 288 20

Teleprinter No.

State *(that is, country)* of nationality: SE

State *(that is, country)* of residence: SE

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

DESOUSA, Sunita
Astra Biochemicals Pvt Ltd
P.O. Box 8013
Malleswaram
Bangalore 560080
India

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is,country)* of nationality: IN

State *(that is, country)* of residence: IN

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Intellectual Property, Patents
Astra Aktiebolag
S-151 85 Södertälje
Sweden

Telephone No.
+46 8 553 260 00

Facsimile No.
+46 8 553 288 20

Teleprinter No.

☐ **Adress for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

PRAHLAD, Dwarakanath
Astra Biochemicals Pvt Ltd
P.O. Box 8013
Malleswaram
Bangalore 560080
India

This person is:
- [ ] applicant only
- [X] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality: IN

State *(that is, country)* of residence: IN

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [X] the United States of America only
- [ ] the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
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- [ ] the United States of America only
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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
- [ ] the United States of America only
- [ ] the States indicated in the Supplemental Box

- [ ] Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes: at least one must be marked)*:

Regional Patent

- [x] AP **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- [x] EA **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
- [x] EP **European Patent:** AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [x] OA **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- [x] AL Albania
- [x] AM Armenia
- [x] AT Austria
- [x] AU Australia
- [x] AZ Azerbaijan
- [x] BA Bosnia and Herzegovina
- [x] BB Barbados
- [x] BG Bulgaria
- [x] BR Brazil
- [x] BY Belarus
- [x] CA Canada
- [x] CH and LI Switzerland and Liechtenstein
- [x] CN China
- [x] CU Cuba
- [x] CZ Czech Republic
- [x] DE Germany
- [x] DK Denmark
- [x] EE Estonia
- [x] ES Spain
- [x] FI Finland
- [x] GB United Kingdom
- [x] GD Grenada
- [x] GE Georgia
- [x] GH Ghana
- [x] GM Gambia
- [x] HR Croatia
- [x] HU Hungary
- [x] ID Indonesia
- [x] IL Israel
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- [x] IS Iceland
- [x] JP Japan
- [x] KE Kenya
- [x] KG Kyrgyzstan
- [x] KP Democratic People's Republic of Korea
- [x] KR Republic of Korea
- [x] KZ Kazakhstan
- [x] LC Saint Lucia
- [x] LK Sri Lanka
- [x] LR Liberia
- [x] LS Lesotho
- [x] LT Lithuania
- [x] LU Luxembourg
- [x] LV Latvia
- [x] MD Republic of Moldova
- [x] MG Madagascar
- [x] MK The former Yugoslav Republic of Macedonia
- [x] MN Mongolia
- [x] MW Malawi
- [x] MX Mexico
- [x] NO Norway
- [x] NZ New Zealand
- [x] PL Poland
- [x] PT Portugal
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- [x] RU Russian Federation
- [x] SD Sudan
- [x] SE Sweden
- [x] SG Singapore
- [x] SI Slovenia
- [x] SK Slovakia
- [x] SL Sierra Leone
- [x] TJ Tajikistan
- [x] TM Turkmenistan
- [x] TR Turkey
- [x] TT Trinidad and Tobago
- [x] UA Ukraine
- [x] UG Uganda
- [x] US United States of America
- [x] UZ Uzbekistan
- [x] VN Viet Nam
- [x] YU Yugoslavia
- [x] ZW Zimbabwe

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- [x] AE United Arab Emirates
- [x] ZA South Africa
- [ ]

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM ☐ Further priority claims are indicated in the Supplemental Box.

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is: national application: country	regional application:* regional Office	international application: receiving Office
item (1) (13.05.98) 13 May 1998	1019/MAS/98	India		
item (2) (22.06.98) 22 June 1998	9802210-6	Sweden		
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office)* identified above as item(s): (2)

* *Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.*

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA / SE

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority):*

Date (day/month/year)	Number	Country (or regional Office)
29 January 1999	ITS SE98/00642	Sweden

Box No. VIII CHECK LIST; LANGUAGE OF FILING

This international application contains the following **number of sheets**:

request	: 4
description (excluding sequence listing part)	: 9
claims	: 2
abstract	: 1
drawings	: 4
sequence listing part of description	:
Total number of sheets	: 20

This international application is **accompanied by** the item(s) marked below:

1. ☒ fee calculation sheet
2. ☒ separate signed power of attorney
3. ☒ copy of general power of attorney; reference number, if any: GF 4353/98 & 1103/99
4. ☐ statement explaining lack of signature
5. ☒ priority document(s) identified in Box No. VI as item(s): (1)
6. ☐ translation of international application into (language):
7. ☐ separate indications concerning deposited microorganism or other biological material
8. ☐ nucleotide and/or amino acid sequence listing in computer readable form
9. ☒ other *(specify)*: ITS SE98/00642

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: English

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Södertälje, 4 May 1999

Susanne Ås Sivborg
Intellectual Property, Patents, Astra Aktiebolag

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1. Date of actual receipt of the purported international application:	2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority (if two or more are competent): ISA /	6. ☐ Transmittal of search copy delayed until search fee is paid.

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Date of receipt of the record copy by the International Bureau:

11619936 BIOSIS NO.: 199800401917
Synthesis of a radioiodinated park nucleotide analog: A new tool for antibacterial screen development.

AUTHOR: Eid Clark N(a); Nesler Michael J; Zia-Ebrahimi Mohammad; Wu Chuyn-Yeh Ernie; Yao Raymond; Cox Karen; Richardson John
AUTHOR ADDRESS: (a)Bristol-Myers Squibb, 5 Research Parkway, Wallingford, CT 06492-7660, USA

JOURNAL: Journal of Labelled Compounds and Radiopharmaceuticals 41 (8):p 705-716 Aug., 1998
ISSN: 0362-4803
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: The Park nucleotide is an important biological building block used in the construction of bacterial cell walls. Herein, we describe the synthesis of a radiolabeled Park nucleotide analog, p-iodophenoxyacyl-Ala-(D)-iso-Glu-Lys-(D)-Ala-(D)-A:a-OH-(125I), using electrophilic destannylation. Anti-Park nucleotide antibody binding assays using a scintillation proximity assay (SPA) system showed good recognition of the radiolabeled surrogate. This methodology could be used for establishing a screen to identify inhibitors of peptidoglycan biosynthesis.